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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Method for extraction and concentration of hydrophilic compounds from hydrophobic liquid matrices

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Method for Extraction and Concentration of Hydrophilic Compounds From Hydrophobic Liquid Matrices

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Method for Extraction and Concentration of Hydrophilic Compounds From Hydrophobic Liquid Matrices

This invention relates to extraction and concentration of hydrophilic compounds, biological materials or particles dispersed or distributed in hydrophobic liquid matrices for the purpose of detection and /or quantification of such contaminants. This invention relates furthermore to capture solutions which improve recovery of such compounds.

Background of the invention

Small amounts of hydrophilic compounds (such as ATP, NAD, NADP, NADH, NADPH, enzymes, free fatty acids, preservatives, biocides, salts) as well as micro-organisms or other particles are often dispersed or distributed in hydrophobic liquid matrices such as crude oil, vegetable oil, petrol and kerosene. Such compounds or particles may constitute a contamination or adulteration or may be additives and preservatives of which a specific concentration is required. It may therefore be desirable to detect and/or quantify such compounds, to establish whether a particular product is safe and appropriate for a particular use. The hydrophilic compounds mentioned above are summarized in the present description as hydrophilic compounds.

By performing an aqueous extraction the hydrophilic compounds can be separated from the hydrophobic matrix and detection / quantification can then be performed on the aqueous extract. To obtain a high recovery in the extraction it is important to obtain good dispersion of the aqueous extractant throughout the hydrophobic matrix. To obtain a low detection limit it is important to keep the ratio of extractant to matrix low. To obtain a fast recovery it is important to get a rapid phase separation after the

extraction step. By using a capture solution according to the invention the recovery of hydrophilic compounds is greatly improved.

Description of the invention

The present invention relates to a method of extraction of hydrophilic compounds dispersed or distributed in a hydrophobic/non-polar/non-ionic liquid matrix. The present invention relates also to a capture solution.

The object of the present invention is a method for extraction and concentration of hydrophilic compounds dispersed or distributed in hydrophobic liquid matrices comprising the following steps:

a) providing a sample of a hydrophobic liquid
b) adding an aqueous capture solution containing an extractant to said sample
c) mixing said sample and said capture solution thoroughly
d) allow the aqueous phase separate from the sample phase
e) measure the compound in the aqueous phase.

Another object of the invention is a capture solution containing an extractant. In preferred embodiment the exctrant is a amphophilic surfactant. In especially preferred embodiments the amphophilic surfactant is a lecithin. In most preferred embodiments the capture solution contains a water-soluble dye or fluorescent compound. For better visibility of the aqueous phase a water-soluble dye like methyleneblue is added to the capture solution.

Figure 1 depicts the procedure according to the present invention. Details are given in the examples. The steps shown are: (1) One liter of sample is collected; (2) the capture solution is added; (3) the mixture is shaken vigorously for 10 seconds; (4) the mixture then is left standing for 5 minutes; (5) the capture solution is collected; (6) the capture solution is

added to a HY-LITE ® pen tube; (7) the capture solution is tested; (8) read HY-LITE ® pen: the emitted light is measured.

Figure 2 shows a comparison of biomass measurements in extracts of kerosene using the extraction method according to the invention. Count of viable cells (x-axis) are compared to the luminometric determination of ATP (y-axis). Experimental details are described in example 3.

The capture solution used according to the present invention is an aqueous solution of an extractant. This capture solution optionally may contain acids, bases or buffers as additive for maintaining a defined pH and/or neutral salts in order to maintain a given ionic strength. A water-soluble dye or fluorescent compound can be added in order to improve the visibility of the aqueous phase. In this document the term water-soluble dye represents both dyes and fluorescent compounds, unless otherwise stated. Methylene-blue is an example for such a water-soluble dye. The concentration of the water-soluble dye is chosen to allow good visibility of the aqueous phase. The extractant is basically a surfactant or emulsifyer. A large variation of tensids and emulsifyers are known in the art, examples are shown in the following table:

type of surfactants / emulsifyer	naturally occurring	derivatives of natural products	synthetic
non-ionic	diglyerides	polyethoxy sorbitantesters	polyethoxymono-alkylarylethers
anionic	cholic acid; inositol-phosphatides	sulfonated recinolesters	sodium laurylsulfate
cationic			benzalkonium-chloride
amphoteric, zwitterionic	lecithins, cephalins;		

Beyond the classification given above, tensids and emulsifyer can be categorized by the balance of their hydrophilic-lipophilic properties (HLB-

value), or by their critical micellar concentration (CMC), or by their solubility in water.

The most preferred extractants belong to the class of amphoteric or zwitterionic surfactants. Within this group lecithins are the most preferred ones. A capture solution according to the invention can optionally contain more than one extractant. The concentration of the most preferred extractant (lecithin) used in capture solutions is preferrably between 0.1% (w/v) and 1% (w/v). Using other detergents as extractant preferred ranges depend on HLB-value and critical micellar concentration, as well as solubility in water.

A low ratio (1:10 or less; preferred: 1:100 or less; mostly preferred 1:1000 or less) of an aqueous capture solution containing an extractant is added to the sample to be tested. The lower limit for the volume ration of the capture solution is given by the solubility of water in the sample, i.e. the volume ratio has to be large enough so that an aqueous phase can be separated from the bulk of the hydrophobic sample. Capture solution and sample is mixed to disperse the capture solution throughout the sample.

The resultant mixture is allowed to settle for a period of time to obtain phase separation. If necessary, the phase separation can be promoted by addition of a further small amount of ionic compounds (typically salt, acid or base) to break the emulsion or by physical treatment (e.g. temperature change, vibration, centrifugation).

After phase separation, the aqueous phase is retrieved and detection of the compounds, biological materials and particles can be performed as desired on the isolated aqueous phase. Methods for this purpose are known in the art. Examples are: measuring ATP by luminometry using luciferase, measuring NAD and/or NADP by cycling reactions, determining the cell count of microbes by plating defined volumes onto a suitable solid growth

medium supporting the growth of the microbe to be counted. WO 02/22854 discloses a cycling reaction scheme for measuring NAD and/or NADP. For the determination of enzyme activities spectrophotometric procedures are known. Toxins, antibiotics, or growth inhibitors can be determined using biological tests like the radial diffusion test using susceptive bacteria as test organism.

Separation times may vary and may be accelerated by use of containers with smooth, hydrophobic/non-polar/non-ionic inner walls. Containers having a conical bottom allow collecting the aqueous lower phase more easily.

The extractant according to the present invention is prepared by dissolving the ingredients in distilled water.

Literature References:

Institute of Petroleum. Standard IP 385/99: Determination of the viable aerobic microbial content of fuels and fuel components boiling below 390°C – Filtration and culture method

IATA: Guidance Material on Microbiological Contamination in Aircraft Fuel Tanks. 1st Edition, Effective 1 December 2002

Examples

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The preferred specific embodiments and examples are, therefore, to

be construed as merely illustrative, and not limitative of the disclosure in any way whatsoever.

The entire disclosures of all applications, patents, and publications cited above and below are hereby incorporated by reference.

The following examples represent practical applications of the invention.

Example 1: Improvement of extraction efficency using capture solution (extraction of ATP)

The use of the current invention to demonstrate improved extraction of free chemical from a hydrophobic liquid sample is illustrated in table 1. The chosen sample was diesel and the marker for free chemical was ATP. 10ml of diesel fuel was transferred to each of two bottles. 1µl of 1.0×10^{-4}M ATP solution was added to each of the bottles and mixed on vortex mixer for 60 seconds. 1ml of water only was added to one bottle and 1ml of capture solution (0.1%(w/v) Lecithin) was added to the other bottle. Both bottles were mixed on vortex mixer for 10 seconds. Bottles were left to stand for 30 minutes before removing settled water and capture solution. Free ATP in recovered water and capture solution was measured by assay using Merck KGaA bioluminescence reagents. 100% recovery was determined by adding 1µl of 1.0×10^{-4}M ATP directly to 1ml of water or 1ml of capture solution and measuring free ATP.
% recovery was calculated,

$$\% \text{ recovery} = \frac{\text{free ATP measured in water or capture solution}}{\text{free ATP measured from 100\% recovery}} \times 100$$

Table 1:

extractant	% recovery
water	3
capture solution (0.1%(w/v) Lecithin)	70

This result shows that capture solution is by far better than water alone for ATP extraction and concentration of ATP from diesel.

Example 2: Improvement of extraction efficency using capture solution (extraction of bacteria using ATP as marker)

The use of the current invention to demonstrate capture and subsequent measurement of biomass components in a hydrophobic liquid sample is illustrated in table 2. The chosen sample was aviation fuel and the marker for biomass determination was Adenosine Triphosphate (ATP).

500ml of fresh aviation fuel was measured into each of two containers. 25µl of a bacterial suspension (*Pseudomonas fluorescens*) was added to each container. Both containers were manually mixed by shaking to distribute the bacteria followed by mixing on a roller mixer for 30 minutes and then finally standing for 30 minutes before testing. 0.5ml of water only was added to one container and 0.5ml of capture solution (0.1%(w/v) Lecithin) was added to the other container. Both bottles were mixed by shaking for 10 seconds. Containers were left for 5 minutes before removing the settled water and capture solution with a Pasteur pipette for testing. Total ATP in recovered water and capture solution was measured by assay using Merck KGaA bioluminescence reagents. 100% recovery was determined by adding 25µl of same bacterial suspension directly to 0.5ml of water or 0.5ml of capture solution and measuring total ATP.

% recovery was calculated,

$$\% \text{ recovery} = \frac{\text{free ATP measured in water or capture solution}}{\text{free ATP measured from 100\% recovery}} \times 100$$

Table 2:

extractant	% recovery
water	26
capture solution (0.1%(w/v) Lecithin)	61

This result shows that capture solution is better than water alone for total ATP (biomass) extraction and concentration from aviation fuel.

Example 3: Extraction of cells according to the present invention and determination of biomass comparing viable cell count and ATP determination

The use of the current invention to demonstrate capture and subsequent measurement of free chemical and biomass components in a hydrophobic liquid sample is illustrated in table 3.

The chosen sample was aviation fuel and the marker for free chemical and biomass determination was Adenosine Triphosphate (ATP). ATP levels indicate levels of microbial contamination of the aviation fuel. Aviation fuel collected from aeroplane wing tanks was used as sample to measure ATP levels and TVC (total viable count). Fuel samples were processed as described below so that an aqueous capture solution could be tested for ATP levels and TVC.

1.0ml of capture solution (0.1%(w/v) Lecithin) was added to approximately 1.0L of fuel sample, fuel and capture solution were mixed by manual shaking for 10 seconds, sample was allowed to stand for 5 minutes before removing the settled capture solution with a disposable Pasteur pipette, capture solution was tested for ATP and TVC (figure 1 shows protocol for measuring ATP).

Free ATP (extracellular ATP) and Total ATP (biomass ATP + extracellular ATP) levels were measured with the HY-LiTE® ATP luminescence assay manufactured by Merck KGaA. To measure free ATP 28µl of capture solution was pipetted into the HY-LiTE® pen (via the reagent cap) and light emission (expressed as RLU – relative light units) measured in the HY-LiTE® luminometer. Total ATP was measured by sampling the capture solution with the HY-LiTE® pen following manufacturer's instructions and light emission measured in the HY-LiTE® luminometer. TVC was determined by plating out 100µl of neat, 10^{-2}, and 10^{-4} dilutions of capture solution on Tryptone Soy Agar plates. Plates were incubated for 2 days at 20°C before counting colonies. Colony forming units (cfu/ml) per ml were calculated,

cfu/ml = no.of colonies x dilution factor x 10

Table 3:

sample	Free ATP (RLU)	Total ATP (RLU)	TVC (cfu/ml)
1	1400	43000	7.1×10^6
2	210	57000	1.42×10^7
3	560	68000	1.35×10^7
4	210	32000	6.6×10^6
5	69	870	1.42×10^5
6	76	6000	3.0×10^5
7	81	8500	1.5×10^6
8	30	16000	4.7×10^6
9	380	19000	5.0×10^6
10	91	3200	8.5×10^5
11	340	88000	1.0×10^7
12	100	33000	9.6×10^6
13	36	76	0.0×10^0
assay back-ground	56	56	---

Data shows that free ATP and total ATP can be extracted and concentrated from the hydrophobic phase into the capture solution at levels significantly different from assay background. Comparing the biomass ATP and the TVC one observes an excellent correlation showing that the microbes have been extracted as viable cells in reproducible yield. The correlation data are:

$r = 0.8872$ $y = 0.0049293 x + 676,7$

The data are presented in figure 2.

Claims

1. A method for extraction and concentration of hydrophilic compounds dispersed or distributed in hydrophobic liquid matrices comprising the following steps:

a) providing a sample of a hydrophobic liquid;

b) adding an aqueous capture solution containing an extractant to said sample;

c) mixing said sample and said capture solution thoroughly;

d) allow the aqueous phase separate from the sample phase;

e) measure the compound in the aqueous phase.

2. An aqueous capture solution containing at least one extractant.

3. A capture solution according to claim 2, wherein said extractant is an amphoteric surfactant.

4. A capture solution according to claim 3, wherein said extractant is a lecithin.

5. A capture solution according to one of the claims 2 to 4 containing a water-soluble dye in an amount to allow good visibility of the aqueous phase.

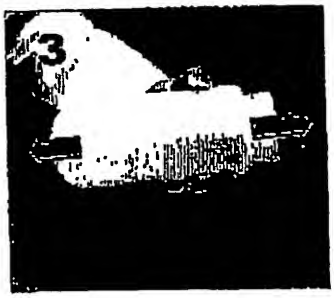
Abstract

Methods for extraction and concentration of hydrophilic compounds, biological materials or particles dispersed or distributed in hydrophobic liquid matrices are disclosed. In order to improve yield the extraction is carried out using a capture solution comprising an extractant. The extract is well suited for the purpose of detection and /or quantification of contaminants in hydrophobic liquids. The preferred extractants are amphiphilic surfactants, e.g. lecithins.

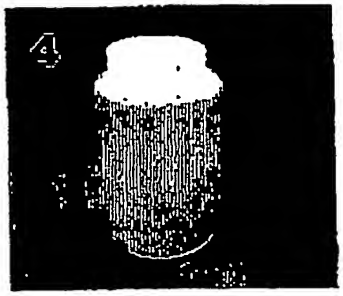
Fig. 1

Collect 1liter fuel

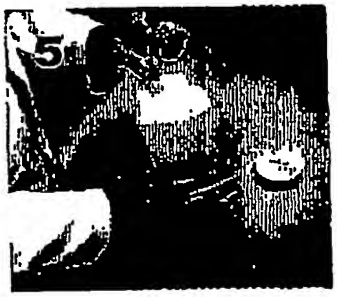
Add Capture Solution



Shake vigorously for
10 seconds

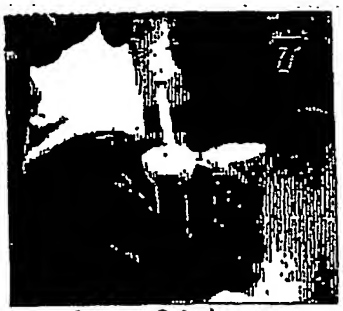


Stand for 5 minutes

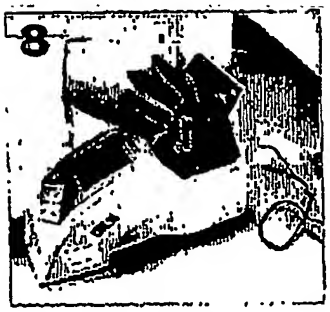


Collect Capture Solution

Add Capture Solution
to HY-LiTE Pen Tube



Test Capture Solution
using HY-LiTE Pen



Read HY-LiTE Pen
in Luminometer

Fig. 2